

Response to immune complex vaccine in chronic hepatitis B patients is associated with lower baseline level of serum IgG galactosylation

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Abstract

The composition of glycan in immunoglobulin G (IgG) has shown to affect various diseases and can be regulated by drugs and preventive vaccination. A hepatitis B surface antigen (HBsAg)-hepatitis B immunoglobulin (HBIG) immune complex (YIC) therapeutic vaccine for chronic hepatitis B (CHB) patients has undergone clinical trials. To explore for markers of CHB, which could be associated with responsiveness to YIC therapeutic vaccine, serum IgG glycosylation in CHB patients was analyzed.

Kinetic changes of serum galactosylated IgG in 53 hepatitis Be antigen (HBeAg)-positive CHB patients treated with YIC were monitored by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) analysis. Whole blood cytokines were assayed by cytokine binding assay kits. All samples were back assayed before treatment, during therapy and follow-up for 6 months from a previous completed clinical trial.

During YIC treatment, 26 patients with lower IgG galactosylation level at baseline [galactosylation level (Gal-ratio) = -0.29, 0.18 (mean, SD)] showed sustained increase of serum galactosylated IgG, and responded to YIC treatment by HBeAg seroconversion. While those who did not respond to YIC treatment [Gal-ratio = -0.40, 0.15 (mean, SD)] failed to show similar changes. Furthermore, this kinetic increase of galactosylated IgG correlated with marked up-regulated IL-2 level, confirming that effective cellular immune responses have participated in responsiveness.

For HBeAg-positive CHB patients lower serum IgG galactosylation level may serve as an indicator for selecting a suitable subpopulation of candidates for YIC therapeutic vaccination.

Abbreviations: ADCC = antibody-dependent cell-mediated cytotoxicity, ALT = alanine aminotransferase, AUC = area under the curve, CHB = chronic hepatitis B, Fc = fragment crystallizable, G0 = fucosylated biantennary glycan carrying no galactose, G1 = fucosylated biantennary glycan carrying one galactose, G2 = fucosylated biantennary glycan carrying two galactose, Gal-ratio = galactosylation level, GEEs = generalized estimating equations, HBeAg = hepatitis Be antigen, HBIG = hepatitis B immunoglobulin, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, IgG = immunoglobulin G, MALDI = matrix-assisted laser desorption/ionization, MS = mass spectrometry, QIT = quadrupole ion trap, ROC = receiver operator characteristic, TOF = time of flight, YIC = hepatitis B surface antigen-hepatitis B immunoglobulin immune complex.

Keywords: chronic hepatitis B, galactosylation, hepatitis Be antigen seroconversion, immunoglobulin G, therapeutic vaccine

Editor: Gunjan Arora.

Jing Han and Guo-Zhong Gong contributed equally.

We gratefully acknowledge financial support from the National Key Research and Development Program of China (2018YFC0910300 & 2016YFA0501303) and National Natural Science Foundation of China (31770858), and National Science and Technology Project of Major Infectious Diseases (2008ZX10002-003 & 2012ZX10002002004, 2017ZX10202201002) and Zhixian Biotech.

The authors declare no conflict of interests.

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Medicine (2019) 98:26(e16208)

Received: 30 November 2018 / Received in final form: 29 May 2019 / Accepted: 3 June 2019

<http://dx.doi.org/10.1097/MD.00000000000016208>

1. Introduction

The fragment crystallizable (Fc) domain of immunoglobulin G (IgG) plays an important immunoregulatory role via binding to its cellular receptors.^[1] The Fc domain displays remarkable heterogeneity, determined both by the amino acid sequence of various IgG subclasses and by the complex Fc-associated N-linked glycan. These determinants regulate the conformational flexibility of the Fc domain and affect its capacity to interact with Fcγ receptors. The glycosylation patterns of IgG have been studied in immune disorders, inflammation, infections, and metabolic diseases.^[2] Furthermore, recent data showed that antibody glycosylation not only is related to disease progression, but also can be manipulated to regulate host immune responses.^[3] Many reports have showed the serum IgG galactosylation characteristics in autoimmune diseases, infectious diseases, and cancers.^[4,5] Fc galactosylation of IgG1 and IgG3 can enhance the complement-dependent cytotoxicity by improving binding of C1q.^[6] Antibody-dependent cell-mediated cytotoxicity (ADCC) was increased in some monoclonal antibodies by Fc galactose.^[7] IgG from the malaria-endemic region adults could inhibit parasite growth by activating ADCC with natural killer cells.^[8] IgG deficient of galactose has a close correlation with inflammation.^[9] HIV-specific antibodies agalactosylation was associated with enhanced Fc-mediated reduction of viral replication, resulting robust antiviral activity.^[10] These findings provide promising clues for improvement of the current therapeutics and for the development of new therapeutics and novel vaccines.

Viral hepatitis B is an infectious disease caused by hepatitis B virus (HBV). Belonging to the hepadnaviridae, with a small circular DNA genome, HBV replicates via an RNA intermediate and secretes huge amount of small envelope protein particles [hepatitis B surface antigen (HBsAg)].^[11] Notwithstanding the tremendous success of preventive vaccine in control of viral hepatitis B, there are still around 300 million individuals infected with HBV, and the number of chronic hepatitis B (CHB) patients may attain 20 million worldwide. Aimed at restoring the immune responses in CHB patients, we have developed a HBsAg-hepatitis B immunoglobulin (HBIG) immune complex (YIC) therapeutic vaccine for CHB patients, and these clinical trial studies have been published elsewhere.^[12-14] To explore individualized effective treatment by YIC, the kinetic changes of IgG galactosylation in responsive and non-responsive hepatitis B antigen (HBeAg)-positive patients under YIC treatment were analyzed. Serum samples from the phase III clinical trial^[12] were back assayed for glycosylation of their IgG in relation to responsiveness to therapeutic vaccine treatment.

2. Materials and methods

Formal approvals from the ethics committees in 21 evaluation centers were completed, and enrollment of patients started at the end of October 2007. A signed written informed consent for participation in this trial was obtained from each patient prior to enrollment.

2.1. Patients

As reported previously, a double-blind, randomized, and placebo-controlled trial was conducted to determine the efficacy of therapeutic vaccine (YIC) for chronic hepatitis B patients who

were aged 18 to 65 years, HBsAg and HBeAg positive for at least 6 months. Study design was published elsewhere.^[12] Briefly, all participants allocated in the YIC group were administrated with 12 intramuscular injections at 4-week interval, with an 8-week break between the 6th and 7th injections, the patients were followed-up for 24 weeks after the termination of immunization. Serum samples were collected at week 0, 12, 24, 40, 52, and 76 after initial injection.^[12]

In this study, 53 patients were enrolled from the YIC treatment group. At the end of follow-up, 26 patients reached the primary endpoint (HBeAg seroconversion). Another 27 patients (non-responders) from the same group whose HBeAg did not convert were selected randomly as controls by matching the age, gender, and genotype. Serum samples were back assayed at 3 time points, namely, week 0 (baseline), week 52 (completion of treatment with YIC for 12 injections), and week 72 (followed for 6 months), with informed consent of utilizing the serum samples for further analysis. Responder (HBeAg seroconversion) was defined as loss of HBeAg, and appearance of anti-HBe at the end of follow-up.

2.2. Isolation of IgG

IgG from blood serum sample was isolated by Protein A Spin Plate for IgG Screening (Thermo Fisher Scientific, Rockford). The isolation was manipulated according to the manufacturer instructions.

2.3. Release and purification of IgG N-glycan

As reported previously,^[15] IgG N-glycan was released by mixing denatured portion of IgG-containing fractions of Protein A column elution and PNGase F (New England Biolabs, Inc., USA) with incubation for 12 hours at 37°C. The released oligosaccharides were subsequently purified using a porous graphitic carbon-containing 96-well plate for mass spectrometry (MS) analysis.

2.4. Matrix-assisted laser desorption/ionization (MALDI) MS analysis

The collected N-glycan samples (0.5 μl) were deposited onto a standard MALDI plate. Then, 0.5 μl of matrix solution, 10 mg/ml 2,5-dihydroxybenzoic acid (Sigma-Aldrich, Germany) in 0.1% (v/v) trifluoroacetic acid in 50% acetonitrile/H₂O (v/v), was added onto the sample layer, followed by re-crystallization to form homogeneity of the spot surface with ethanol. Each sample was spotted in triplicate. The samples were interrogated automatically in a “batch mode” by AXIMA Resonance MALDI-quadrupole ion trap (QIT)-time of flight (TOF) MS (Shimadzu Corp. JP) equipped with a 337 nm nitrogen laser in reflector positive ionization mode. The m/z range was monitored to span from 500 to 5000. Tandem mass spectrometry was utilized to validate the component of the detected glycans. The GlycoWorkbench software was used for the annotation of MS spectra. The MALDI MS spectra data were pre-processed, normalized and extracted using the software of Progenesis MALDI before further analysis.

2.5. Whole blood cytokines assay

Whole blood samples from 53 patients at baseline, 52 and 76 weeks were assayed for IL-2, IL-4, IL-6, TNF-α, interferon-γ, and IL-17A by FACS Calibur (BD Biosciences, Sunnyvale, CA, USA),

using cytokine binding assay kits (BD PharMingen, BD Biosciences, USA), which has been published recently.^[16]

2.6. Data management and statistical analysis

SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA) and GraphPad Prism 5 were used for analysis in this study. HBV DNA, HBsAg, HBeAg, IgG galactosylation and alanine aminotransferase (ALT) levels of responders and non-responders were compared at baseline, week 52 and week 76. For binary data, the Chi-Square test, or Fisher exact test were employed as appropriate. For dichotomous outcomes, such as HBV DNA, HBsAg, HBeAg, and IgG galactosylation level, ANOVA was used after logarithmically transform. Wilcoxon rank-sum test was applied for the comparison of ALT level.

Repeated measures analysis was performed using a generalized estimating equations (GEEs) method to adjust the dependence among repeated observations made on the same patient while testing the group (responder and non-responder) and time effects as well as the potential interaction between genotype, gender, group and time.^[17] The unstructured working covariance matrix which provided robust estimation of covariance to the structure was applied. Adjustments were made for group, gender, genotype, age, level of HBV DNA, HBsAg, and HBeAg.

To explore the potential prediction value of IgG galactosylation level at baseline with regard to the outcome after YIC treatment, which was HBeAg seroconversion, the receiver operator characteristic (ROC) curve as well as Youden index method was applied to define the optimal cutoff of Gal-ratio at baseline. Hereafter, the sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio, as well as 95% confidence interval for sensitivity and specificity^[18] were calculated. A P -value $< .05$ (2-tailed) was considered as statistically of significance.

3. Results

3.1. Profiling serum IgG N-glycan by MALDI MS analysis

Using a simple and accurate method for the relative quantification of serum IgG galactosylation in our previous study,^[19] serum IgG N-glycan profile from each CHB patient was identified. A representative MALDI-QIT-TOF MS spectrum model displaying the N-glycoforms of serum IgG from CHB patients is shown in Figure 1. The most abundant components are fucosylated biantennary glycans, including G0 (carrying no galactose), G1 (carrying 1 galactose), and G2 (carrying 2 galactose) as shown in the MS spectrum. The IgG galactosylation level (referred to as Gal-ratio) was measured by calculating the relative intensities of agalactosylated (G0) vs monogalactosyl (G1) and digalactosyl (G2) N-glycans according to the formula of $G0/(G1+G2 \times 2)$ as described previously.^[19]

3.2. Virological and clinical characteristics between responders and non-responders

These characteristics were extracted from our previous publication.^[12] Of the 53 patients, 27 were infected with HBV genotype B, while other 26 were infected with genotype C. There was no significant difference in age, gender, and distribution of genotype B and C between responders and non-responders ($P > .05$) (Table 1). At week 0, levels of serum HBsAg, HBV DNA, and ALT were comparable between responders and non-responders. At the end of YIC treatment, decrease in HBV DNA and conversion from HBeAg positive to negative were observed in responders, compared to non-responders. At the end of follow-up, all responders approached HBeAg seroconversion to anti-HBe positive, ALT normalization and an average of 0.6 \log_{10} decrease of HBsAg were only observed in the responders (Table 1).

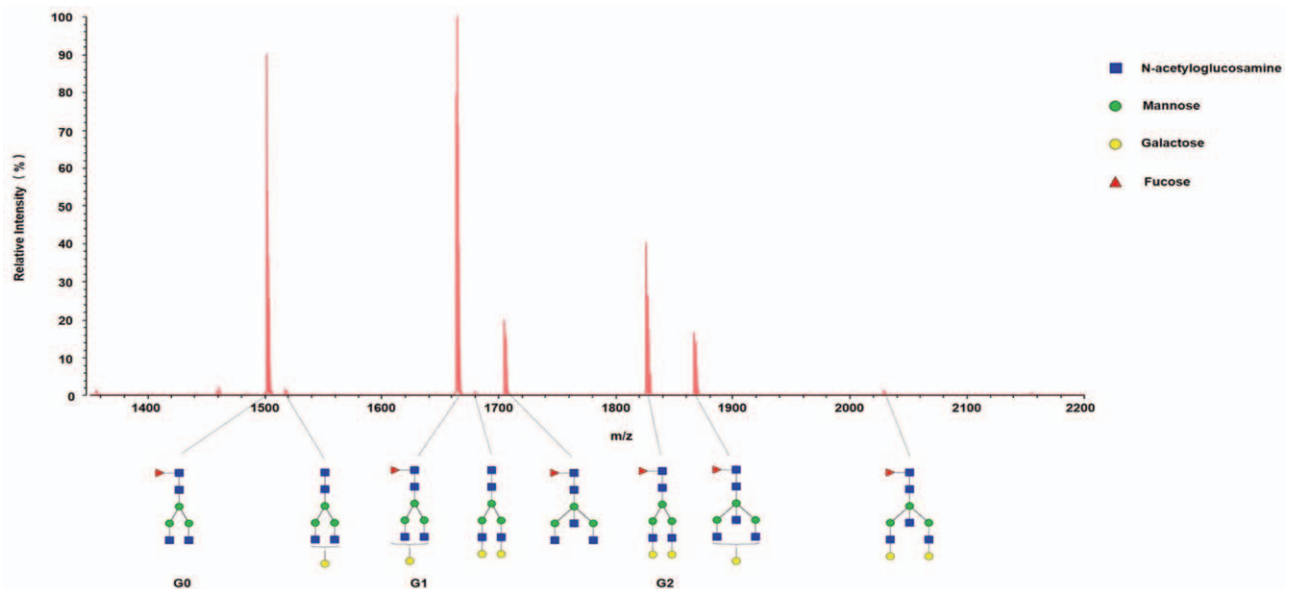


Figure 1. Representative MALDI-QIT-TOF MS spectrum of serum IgG N-glycan profiles from CHB patients. In the mass spectrum, the x-axis (m/z) and the y-axis (relative intensity) represent mass-to-charge ratio and relative signal intensity of the IgG N-glycans, respectively. The annotation of peaks on mass spectrum was based on the relative molecular weight of IgG N-glycans. IgG N-glycans are comprised of N-acetylglucosamines, mannose, galactose, and fucose residues. Structure abbreviations: G0, G1, and G2 indicate fucosylated biantennary glycans with no galactose, 1 galactose, and 2 galactose, respectively.

Table 1
Clinical and virological characteristics between responders and non-responders.

Variables	HBeAg ⁺ seroconversion at week 76		P value
	Responders N=26	Non-responders N=27	
Age, year [mean (SD)]	25.3 (5.0)	27.0 (5.4)	>.05
Male [n (%)]	18 (69.2)	24 (88.9)	>.05
Genotype B [n (%)]	13 (50.0)	14 (51.9)	>.05
Week 0			
ALT [†] , IU/liter [mean (SD)]	185.8 (85.9)	184.3 (95.4)	>.05
HBsAg [‡] quantification-log ₁₀ , IU/ml [mean (SD)]	4.0 (0.5)	4.2 (0.6)	>.05
HBeAg [*] semi-quantification-log ₁₀ , S/CO value/ml [mean (SD)]	2.7 (0.4)	2.9 (0.2)	.03
HBV [§] DNA- log ₁₀ , IU/ml [mean (SD)]	6.1 (0.8)	6.4 (0.7)	>.05
Week 52			
ALT [†] , IU/liter [mean (SD)]	158.7 (236.5)	169.8 (128.3)	>.05
HBsAg [‡] quantification-log ₁₀ , IU/ml [mean (SD)]	3.5 (0.6)	4.0 (0.5)	<.05
HBeAg [*] semi-quantification-log ₁₀ , S/CO value/ml [mean (SD)]	0.7 (1.0)	2.4 (1.8)	<.001
HBV [§] DNA- log ₁₀ , IU/ml [mean (SD)]	4.5 (1.6)	5.7 (1.5)	<.05
Week 76			
ALT [†] , IU/liter [mean (SD)]	31.0 (25.9)	177.2 (187.0)	<.001
HBsAg [‡] quantification-log ₁₀ , IU/ml [mean (SD)]	3.4 (0.5)	3.8 (0.6)	<.05
HBeAg [*] semi-quantification-log ₁₀ , S/CO value/ml [mean (SD)]	-0.3 (0.1)	2.2 (0.9)	<.001
HBV [§] DNA- log ₁₀ , IU/ml [mean (SD)]	3.1 (1.0)	5.5 (1.6)	<.001

* HBeAg = hepatitis Be antigen.

† ALT = alanine aminotransferase.

‡ HBsAg = hepatitis B surface antigen.

§ HBV = hepatitis B virus.

3.3. Increase of IgG galactosylation in responders during YIC treatment

During YIC treatment, a sustained and dramatic increase of IgG galactosylation was found in responders versus non-responders (Fig. 2). The increase continued after cessation of treatment even in the follow-up 6 months ($P=.02$). Interestingly, significant statistical higher IgG Gal-ratio was found in responders $[-0.29, 0.18$ (mean, SD)] than that in non-responders $[-0.40, 0.15$ (mean, SD)] even at baseline ($P=.02$). IgG Gal-ratio difference was not statistically significant between responders and non-responders at 72 weeks. After adjusting the age, gender, HBV genotype, as well as HBV DNA, HBsAg, and HBeAg levels at baseline, the pattern of differences in IgG galactosylation between

responders and non-responders was statistically significant ($P<.001$) (Table 2). In addition, increase of IgG galactosylation was more marked in responsive HBV genotype B patients compared to those in genotype C patients (Table 3).

3.4. Increase of IgG galactosylation with upregulation of IL-2 in responders

To explore the association between levels of IgG galactosylation and cytokine levels in patients, correlation analysis was carried out using IgG galactosylation levels and cytokine levels at baseline and at the end of follow-up (76 weeks). Only at the end of follow-up (week 76), among the 6 cytokines monitored, IL-2 significantly negatively correlated with levels of IgG Gal-ratio, while IL-17A also showed substantial negative correlation (IL-2: $r=-0.54$, $P<.002$; IL-17A: $r=-0.41$, $P=.04$; respectively).

3.5. Association between IgG galactosylation at baseline and HBeAg seroconversion

The optimal cutoff Gal-ratio value to predict HBeAg seroconversion at week 76 in HBeAg-positive CHB patients was defined by receiver operator characteristic (ROC) curve (Fig. 3). The area under the curve (AUC) of ROC reached 0.691 ($P=.02$). A cutoff Gal-ratio value of 0.60 was calculated by Youden index method. By this cutoff, a satisfied performance of prediction for HBeAg seroconversion after YIC treatment was concluded, with a sensitivity of 42% (95% CI: 23–61%) and a specificity of 93% (95% CI: 83–100%). In particular, the positive predictive value was quite promising, which reached 85%. The accuracy was 0.68, and negative predictive value, positive likelihood ratio, and negative likelihood ratio were 63%, 5.7 and 0.62, respectively.

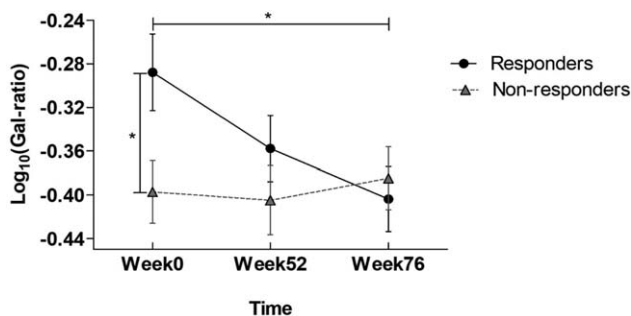


Figure 2. The kinetic trend of IgG galactosylation level in responders and non-responders. Comparison of Gal-ratio (\log_{10} transformed) values at week 0, week 52, and week 76 in the responders and non-responders. P value for comparison between week 0 and week 76 in responders was .02 from paired t test. P value for comparison between responders and non-responders at week 0 was .02 from 2-tailed independent t test.

Table 2**The coefficient of generalized estimating equation in estimating the trend of IgG* galactosylation.**

Parameter	Estimate	95% confidence limits	P value
Intercept	-0.29	-0.70, 0.11	.15
Time (week)	-0.02	-0.06, 0.03	.44
Group (responders vs non-responders)	0.18	0.08, 0.29	<.001
Gender (male vs female)	0.06	-0.08, 0.21	.39
HBV [†] genotype (B vs C)	0.02	-0.12, 0.15	.82
Age (year)	-0.00	-0.01, 0.00	.21
HBV [†] DNA at baseline (log ₁₀ , IU/ml)	-0.05	-0.11, 0.02	.17
HBsAg [‡] quantification at baseline (log ₁₀ , IU/ml)	0.01	-0.12, 0.14	.91
HBeAg [§] semi-quantification at baseline (log ₁₀ , S/CO value/ml)	0.06	-0.03, 0.15	.20
Time*genotype	0.01	-0.02, 0.05	.45
Time*gender	0.03	-0.02, 0.07	.27
Time*group	-0.06	-0.09, -0.03	<.001

* IgG = immunoglobulin G.

† HBV = hepatitis B virus.

‡ HBsAg = hepatitis B surface antigen.

§ HBeAg = hepatitis Be antigen.

4. Discussion

N-linked glycosylation of IgG is an important posttranslational modification of host proteins. Because of the high abundance of IgG in serum, and the Fc-Fc receptor binding roles in initiating immune responses, IgG glycosylation has been studied in a number of diseases.^[20] In rheumatoid arthritis patients, non-galactosylated IgG is associated with disease status and may act as an indicator for prognosis, and decrease of galactose in IgG is a common feature in various cancers.^[15] Increase of serum IgG level commonly occurs in patients with chronic liver disease and is highly related to disease progression.^[3,21] In addition, CHB patients presented higher galactose deficiency than healthy controls.^[3,21] Recently, studies on glycosylation have been reported in other areas, including nephropathy, breast adenocarcinoma,^[22] colon cancer,^[23] septicemia,^[24] HIV,^[10] metabolic diseases,^[9] and vaccinations.^[20]

As YIC therapeutic vaccination is an active immuno-therapy, and its therapeutic efficacy is based on modulating host immune responses via Fc-Fc receptor interactions, the IgG glycosylation of each patient could affect his/her therapeutic responsiveness. By analysis of dynamic changes of IgG glycosylation in YIC treated patients in association with cytokines, the increase in serum IgG galactosylation was highly correlated with up-regulation of IL-2 expression in responsive HBeAg-positive CHB patients. This observation indicates that modulation in IgG glycosylation is in

association with regulation of immune responses in CHB patients. IL-2 as the T cell growth factor has pleiotropic natures and multiple functions. The up-regulation of IL-2 supports that YIC therapy was functioned through accelerated T cell-mediated immune response. This observation is in accordance with our recent immune cellular study, showing that by administration of YIC, significant upregulated IL-2 expression was observed in CD4+ and CD8+ cells, with increase in interferon- γ expression and decrease of inhibitory factors (IL-10 and TGF- β ^[16]). Another recent article reported that changes in glycosylation of IgG in CHB patients treated with antiviral nucleotides were related to changes in TGF- β expression,^[21] which also showed that modification in IgG glycosylation was associated with modified expression of cytokines.

To explore for individualized treatment with YIC, based on this back assay from a previous clinical trial, lower baseline levels of IgG galactosylation in CHB patients may serve as a potential predictor for selecting a subpopulation of CHB patients for YIC therapy. Besides, viral genotype B infected CHB patients may also be considered as another selective parameter for using YIC treatment.

Unlike drug therapy, efficacy of immunotherapy highly depends on individual functional immune status of patients. To practice precision medicine in immunotherapy, evaluation of individual immune status is critical. Two approaches may be employed. One is to do a complete survey of the immune elements in patients before clinical trial and identify the positive and

Table 3**Distribution of IgG* Gal-ratio[†] in two genotype responders of CHB[‡] patients.**

Time	Week 0	Week 52	Week 76	P ¹ value	P ² value
Genotype B (N = 13)	-0.29 (0.12)	-0.39 (0.16)	-0.43 (0.16)	.08	.02
Genotype C (N = 13)	-0.28 (0.23)	-0.32 (0.15)	-0.37 (0.15)	.61	.25

* IgG = immunoglobulin G.

† Gal-ratio = galactosylation level.

‡ CHB = chronic hepatitis B.

Gal-ratio values were log₁₀ transformed.

Data are mean (SD).

P¹-values for comparisons between week 0 and week 52 were from paired t tests.P²-values for comparisons between week 0 and week 76 were from paired t tests.

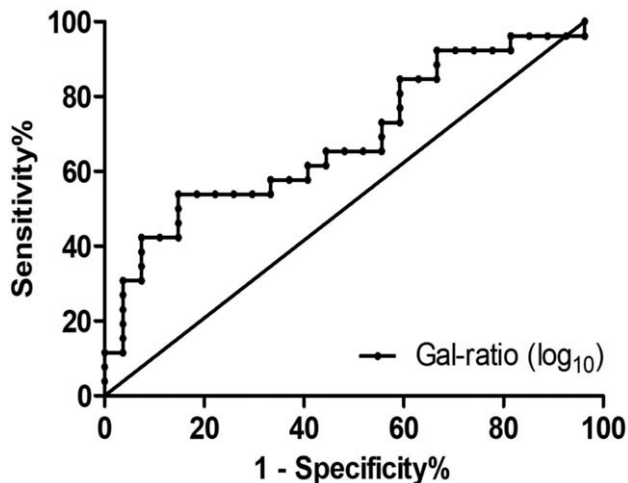


Figure 3. Performance of baseline IgG galactosylation level to differentiate responders from non-responders. ROC curve for baseline IgG Gal-ratio (\log_{10} transformed) values to differentiate responders from non-responders. AUC of ROC=0.691. SE=0.073, $P=.02$. Sensitivity=42% (95% CI: 23–61%). Specificity=93% (95% CI: 83–100%). Cutoff value of Gal-ratio (\log_{10})=−0.219.

negative factors after completion of trial. The other is to use results of completed clinical trials to reveal certain immune elements as potential predictors by back assay. Using the latter approach, we have detected a candidate predictor for favorable responses to YIC treatment.

Although the number of samples stated here was limited, the information provided by these findings is valuable for further investigations. By studying the underlying mechanisms between IgG galactosylation and cytokines will deepen our knowledge on interactions between humoral and cellular immune responses. The increased natural killer cell expression of Fc γ RIIIa and ADCC activity to antibodies is related to the Fc γ RIIIa-158 V/V and V/F polymorphism.^[25] Studying the Fc γ RIIIa polymorphism might help to understand the association between IgG galactosylation and response to YIC therapy in CHB patients in future. Further studies on Fc receptors in CHB patients are necessary, to improve the strategy of using immune complexes or other therapies via Fc for treatment of diseases.

Acknowledgments

We would like to thank the dedicated people of the YIC Efficacy Trial Study Team (Drs. Dao-Zhen Xu, Guo-Zhong Gong, Hong Ren, Li-Min Guo, Ai-Min Sun, Min Xu, Lan-Juan Li, Xin-Hui Guo, Zhen Zhen, Hui-Fen Wang, Huan-Yu Gong, Cheng Xu, Nan Jiang, Chen Pan, Zuo-Jiong Gong, Ji-Ming Zhang, Jia Shang, Jie Xu, Qing Xie, Tie-Feng Wu, Wen-Xiang Huang, Yong-Guo Li) who made this study possible.

Author contributions

Shi-Fang Ren, Guo-Zhong Gong, Yu-Mei Wen, Xuan-Yi Wang and Jian-Xin Gu contributed to the study's conception and design. Guo-Zhong Gong, and Jian-Hua Lei collected the serum samples. Shi-Fang Ren, Jing Han, Jing Xu, Wen-Jun Qin and Rui-Huan Qin carried out the study. Jian-Hua Lei and Xuan-Yi Wang

analyzed the data. Jing Han, Shi-Fang Ren, Yu-Mei Wen and Jian-Xin Gu wrote the paper.

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